American Genetic Association

Journal of Heredity, 2018, 520–529
doi:10.1093/jhered/esy026
Original Article
Advance Access publication 7 June 2018

OXFORD

Original Article

Molecular Insights Into the Ctenophore Genus *Beroe* in Europe: New Species, Spreading Invaders

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Received August 23, 2017; First decision June 2, 2018; Accepted June 5, 2018.

Corresponding Editor: Jose Lopez

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Abstract

The genus *Beroe* Browne, 1756 (Ctenophora, Beroidae) occurs worldwide, with 25 currently-described species. Because the genus is poorly studied, the definitive number of species is uncertain. Recently, a possible new *Beroe* species was suggested based on internal transcribed spacer 1 (ITS1) sequences from samples collected in Svalbard, Norway. Another species, *Beroe ovata*, was introduced to Europe from North America, initially in the Black Sea and subsequently (and possibly secondarily) into the Mediterranean and Baltic Seas. In areas where ctenophores have been introduced, they have often had significant detrimental ecological effects. The potential for other cryptic and/or undescribed *Beroe* species and history of spread of some species in the genus give reason for additional study. When alive, morphological hallmarks may be challenging to spot and photograph owing to the animals' transparency and near-constant motion. We sampled and analyzed 109 putative *Beroe* specimens from Europe, using morphological and molecular approaches. DNA analyses were conducted using cytochrome oxidase 1 and internal transcribed spacer sequences and, together with published sequences from GenBank, phylogenetic relationships of the genus were explored. Our study suggests the presence of at least 5 genetic lineages of *Beroe* in Europe, of which 3 could be assigned to known species: *Beroe gracilis* Künne 1939; *Beroe cucumis* Fabricius, 1780; and *Beroe ovata* sensu Mayer, 1912. The other 2 lineages (here provisionally named *Beroe "norvegica"* and *Beroe "anatoliensis"*) did not clearly coincide with any known species and might therefore reflect new species, but confirmation of this requires further study.

Keywords: cytochrome oxidase (COI), DNA barcoding, gelatinous zooplankton, invasive species, internal transcribed spacer (ITS)

Introduction

The transport of species to new areas outside of their native range has become a major threat to the health of global marine ecosystems (Ruiz et al. 1997; Molnar et al. 2008). Gelatinous zooplankton in the phyla Cnidaria and Ctenophora are among the many groups that have been transported and introduced widely (Costello et al. 2012; Nowaczyk et al. 2016). Many gelatinous zooplankton have life-history characteristics that may make them particularly adept invaders, including high fecundity, rapid growth, diverse diet, reversible development, and encystment (Boero et al. 1996; Piraino et al. 2004). Unfortunately, there exists a dearth of basic information pertaining to gelatinous zooplankton dynamics for many regions (Brotz et al. 2012; Licandro et al. 2015). The lack of data on gelatinous zooplankton is problematic given the substantial impact high abundances and/or introduced gelatinous zooplankton can have on an ecosystem (e.g. Condon et al. 2013). There exist multiple reasons for this lack of data, including the difficulty in sampling often highly dispersed individuals (Purcell 2009), fragility of specimens (Haddock 2007; Licandro et al. 2015), morphological complexity, and limited knowledge of their taxonomy. Thus, though ctenophores are very broadly distributed and found in virtually all marine environments, they remain relatively poorly known (Harbison 1985; Podar et al. 2001).

An example of the ongoing fluidity in ctenophore identification is the introduction of *Mnemiopsis leidyi* A. Agassiz, 1865 to the Black Sea in the 1980s (Vinogradov et al. 1989). Prior to this introduction, 3 species of this genus were described as native to North American waters (*M. gardeni* L. Agassiz, 1860, *M. leidyi*, and *M. mccradyi* Mayer, 1900) (Mayer 1912). After detailed morphological study and analyses of published sources, Seravin (1994) concluded that only *M. leidyi* was legitimate. However, Mills (2018) suggests that *M. gardeni* has precedence, as the oldest name, whereas *M. mccradyi* is a junior synonym of *M. leidyi*.

Once established in the Black Sea, *M. leidyi* spread naturally and via ballast water into the adjacent Azov, Caspian, and Mediterranean Seas (Shiganova et al. 2001). In 2005, it reached the Baltic and North Seas (Javidpour et al. 2006; Boersma et al. 2007) and has continued to spread in both northern and Southern Europe (Boero et al. 2009; Fuentes et al. 2010; Schaber et al. 2011; Antajan et al. 2014).

Several studies have analyzed molecular markers, including internal transcribed spacer (ITS), cytochrome c oxidase subunit I (COI), cytochrome B, and nuclear microsatellite markers to examine species diversity in the genus *Mnemiopsis* and to identify the origin(s) of introduced populations (e.g. Reusch et al. 2010; Ghabooli et al. 2011, 2013; Bolte et al. 2013; Bayha et al. 2014). All genetic studies indicated that introduced populations originated from the Western Atlantic Ocean, with the Southern European population(s) originating from the vicinity of the Gulf of Mexico and Northern European ones from the northeastern coast of the United States.

M. leidyi was eventually joined in Europe by its major predator, the ctenophore *Beroe ovata* (Konsulov and Kamburska 1998). All *Beroe* species play an important ecological role in controlling the abundance of zooplanktivorous ctenophores such as *M. leidyi* (Greve et al. 1976; Mianzan 1999; Shiganova et al. 2014a). Although this introduced *Beroe spp.* was eventually identified as *B. ovata* sensu Mayer both morphologically (Seravin et al. 2002) and using DNA analyses (Bayha et al. 2004), the identification of introduced *B. ovata* was even more complicated than that of *M. leidyi*. Occurring in all oceans, the genus *Beroe* was described by Browne in 1756 and a total of 25 species have been described worldwide (Supplemental Table 1; Mills 2018; WoRMS Editorial Board 2018), although the validity of some species remains unknown (Greve et al. 1976; Harbison 1985; Mills 2018).

Collection and morphological identification of *Beroe* is challenging for a variety of reasons including few reliable or obvious morphological characters for identification (Harbison et al. 1978; Shiganova et al. 2007; Shiganova and Malej 2009; Majaneva and Majaneva 2013), plasticity in body shape, and difficulties in collection and preservation. When preserved, animals lose their original body shape and structure, so identification must be done using live specimens. This involves identification either at the sampling location or after transport to laboratory facilities. Photographs can help to preserve morphological details for the identification process, but the animals' near-constant motion may render photography difficult. Because of these challenges, species within the order Beroida are very poorly studied.

Controversy exists regarding species identity and describer for *B. ovata* (Bayha et al. 2004). In short, Chun (1880) published a study on Mediterranean ctenophores, including a species he called *B. ovata* Eschscholtz (called *B. ovata* sensu Chun in Bayha et al. 2004). Subsequently, Mayer (1912) published a study on American ctenophores, including species he called *B. ovata* Chamisso and Eysenhardt (*B. ovata* sensu Mayer in Bayha et al. 2004) and *B. cucumis* Fabricius, found in Greenland (Fabricius 1780). According to Bayha et al. (2004), *B. ovata* sensu Chun from the Mediterranean is a member of a widespread species that includes *B. cucumis* sensu Mayer from the western Atlantic and eastern Pacific. Due to the taxonomic uncertainties within the genus *Beroe* and inconsistencies involving the original species descriptions, Bayha et al. (2004) used the name *B. ovata* sensu Mayer for *B. ovata* from the western Atlantic and the Black Sea and the name *Beroe cucumis* sensu Mayer (=*B. ovata* sensu Chun) for those found in the Mediterranean, western Atlantic, and eastern Pacific until a thorough systematic revision of the genus *Beroe* could be done (Bayha et al. 2004).

Other *Beroe* reported in the Mediterranean include *Beroe forskalii* Edwards, 1841 (Mills 2018) and *Beroe mitrata* Moser, 1907 (Moser 1907; Tamm and Tamm 1993). Several other species of *Beroe* are also native to European waters, including *B. cucumis* (worldwide distribution, including Denmark, Norway, and the White Sea), *Beroe abyssicola* Mortensen, 1927 (Barents Sea, White Seas), and *Beroe gracilis* (North Sea, Baltic Sea) (Mills 2018). In the Black Sea, introduced *Beroe* spp. was initially identified as *B. cucumis*, possibly a result of discharge of Arctic-sourced ballast water (Zaitzev 1998), or as *B. ovata* from the Mediterranean Sea (Konsulov and Kamburska 1998). Subsequent analyses identified the species conclusively as *B. ovata* sensu Mayer, introduced from the western Atlantic (Bayha et al. 2004). The same species was subsequently recorded in the Baltic Sea in 2012 (Shiganova et al. 2014b).

Given the uncertainty regarding the morphological identification of *Beroe* species and the possibility of undescribed or incorrectly described species, molecular analysis represents one potential tool to begin to clarify some of the species/geography relationships. DNA barcoding of species using species-specific sequences can separate closely related taxa or, conversely, merge allopatric populations into a single species (Hebert et al. 2003). Molecular studies have been used to identify other gelatinous zooplankton (e.g. jellyfish) (Licandro et al. 2015). Barcoding on ctenophores has been conducted using 18s rDNA, ITS1, and mitochondrial cytochrome b (*cytb*) (Podar et al. 2001; Bayha et al. 2004; Bayha et al. 2014).

In this study, we collected, photographed, and morphologically identified live and photographed *Beroe* specimens. Then, we used sequence data from COI, ITS1/ITS2, and published sequences to

explore the phylogenetic relationships of *Beroe* in the seas of Europe and determine whether our classifications based on morphology could be supported by the phylogenetic analysis of DNA sequence data.

Materials and Methods

Sampling and Morphological Identifications

Specimens were opportunistically collected from 9 locations around Europe in association with *M. leidyi* blooms (Figure 1) using hand nets from the surface or while snorkeling or with planktonic Juday net. A total of 109 *Beroe* specimens were included in our analysis (Supplementary Table 2). Prior to preservation in ethanol, alive or photographed individuals were preliminarily identified as *B. ovata* from the Black Sea (Russian and Bulgarian coastal waters) and the Levantine Sea (Israel coastal waters); as *B. cucumis* sensu Mayer from France, and as *B. ovata/B. cucumis* from Norway (Arboretet) and the White Sea following morphological structure descriptions including body shape, ratio of width and length, configuration of aboral organ and mouth, constitution of meridional and paragastral canals, and branching and anastamoses of diverticulae.

Samples were preserved in 95% ethanol for genetic analysis. All samples from this study are stored at the Great Lakes Institute for Environmental Research.

Molecular Analysis

Genomic DNA was extracted from a small portion of tissue using the QIAamp DNA Mini kit with the DNA Purification from Tissues protocol (Qiagen Inc., Toronto, Ontario, Canada). A portion of the COI gene was PCR-amplified using primers F019 (5′-ATTTTCTCTTTACATTTAGCNGG-3') and R021 (5'-CCTAAAAARTGTAAAGGAAA-3′), whereas a fragment including a portion of the 18S ribosomal RNA (rRNA), all of ITS1, the complete 5.8S rRNA, the complete ITS2, and a portion of the 28S rRNA (hereafter called the ITS fragment, or ITS) was PCR-amplified using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′, White et al. 1990). All COI and ITS PCR reactions were performed in 25 µL reactions containing 1.0 µL of template DNA, 2.0 µL 20mM Mg_2SO_4 (Bio Basic Inc., Markham, Ontario, Canada), 0.5 µL 10 mM dNTPs (Bio Basic), 0.5 µL each 10 mM primer, 2.5 µL 10X PCR buffer (Bio Basic), and 0.1 µL 5U/µL Taq polymerase (Bio Basic). Cycling conditions for PCR were: an initial denaturation step of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 50 °C (COI) or 52 °C (ITS) for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 8 min.

Cycle sequencing was conducted using an ABI-PRISM Big Dye terminator cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA) and sequences analyzed on an ABI 3730xl capillary sequencer (Thermo Fisher Scientific). COI and ITS fragment sequences were aligned and edited using Sequencher v.5.0 (Gene Codes Corporation,

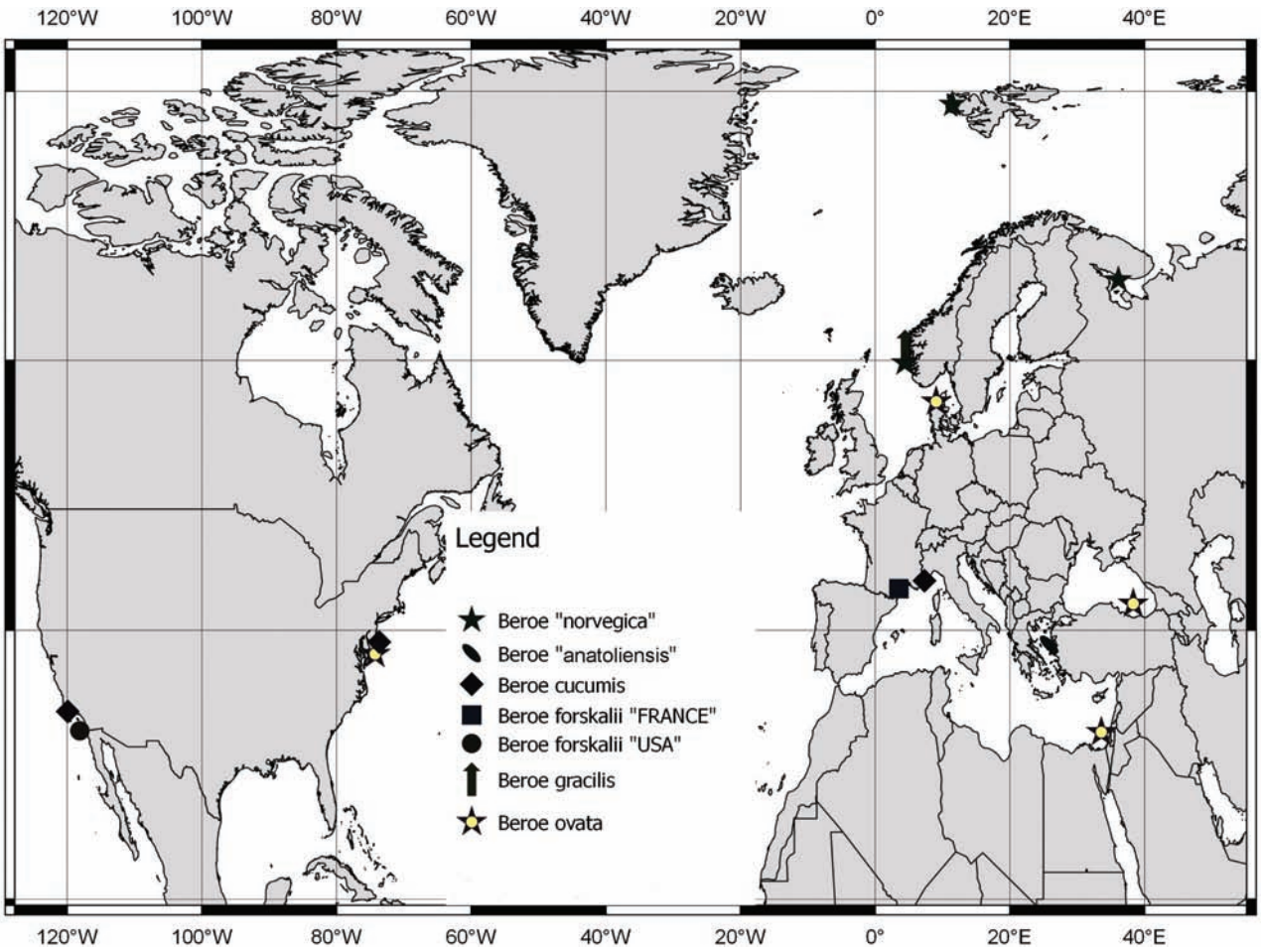

Figure 1. Worldwide distribution of *Beroe* spp. ITS and COI DNA sequences used in this study.

Ann Arbor, MI). Analyses were performed with all sequences separate and with individuals sharing identical sequences grouped under a single sequence identifier (Supplementary Table 2). Alignments were optimized with Geneious v9.1.7 (Biomatters Ltd., Aukland, New Zealand) using the Geneious alignment approach and default settings. We constructed majority-rule consensus neighbor-joining trees using a Tamura-Nei distance model and 1000 bootstrap replicates in Geneious. We also constructed majority-rule consensus maximum-likelihood (ML) trees, using the PhyML 3.2.20160530 plugin in Geneious (Guindon and Gascuel 2003; Guindon et al. 2010). ML analyses were performed for 1000 bootstrap replicates, with a GTR+I+G model, NNI topology search, and optimizing tree topology, branch lengths, and substitution rates. Bayesian analyses were performed using the MrBayes 3.2.6 plugin in Geneious (Huelsenbeck and Ronquist 2001). Bayesian analyses for both markers consisted of 2 runs of 4 chains each with unconstrained branch lengths. Chains were run for 10 000 000 generations, with the first 2 500 000 generations discarded as burn-in and trees sampled every 100 generations. The Bayesian consensus was computed based on 75 000 trees for each marker. Few published *Beroe* spp. sequences are available for either marker, thus only *B. forskalii*, *B. ovata*, and *B. cucumis* sensu Chun (previously *B. ovata* Chun) reference sequences were included in the ITS trees (Figure 2) and only a single *B. ovata* reference sequence was available for inclusion in the COI trees (Figure 3). An additional sequence, from an undescribed *Beroe* sample collected from Svalbard (Majaneva and Majaneva 2013) was included in the ITS analysis. Two *B. forskalii* sequences were used as outgroups in the ITS analysis shown in Figure 2, whereas a single *M. leidyi* sequence was used as an outgroup in the COI analysis shown in Figure 3. Additional phylogenetic analyses (not shown) were performed with all of our unique sequences plus either 18 published *M. leidyi* COI sequences (Accession nos. KF432105.1-KF435121.1, JF760210.1) or 29 published *M. leidyi* ITS sequences (Accession nos. GU062750.1-GU062762.1, HM007193.1-HM007195.1, HM147257.1-HM147269.1) to test for any effect of different outgroups and to facilitate comparisons of genetic differentiation for each marker between our proposed species and a ctenophore species with good representation on GenBank. We also calculated pairwise uncorrected % identities between all our sequences and published *Beroe* sequences, as well as between the *M. leidyi* sequences listed above.

Results

Molecular Analysis

Amplification of the ITS fragment and COI genes was attempted for 109 and 99 individuals, respectively. However, amplifications failed for 2 individuals for the ITS fragment and 10 individuals for COI, leaving 107 individuals in the ITS dataset and 89 individuals in the COI dataset that were used in the phylogenetic analyses (Supplementary

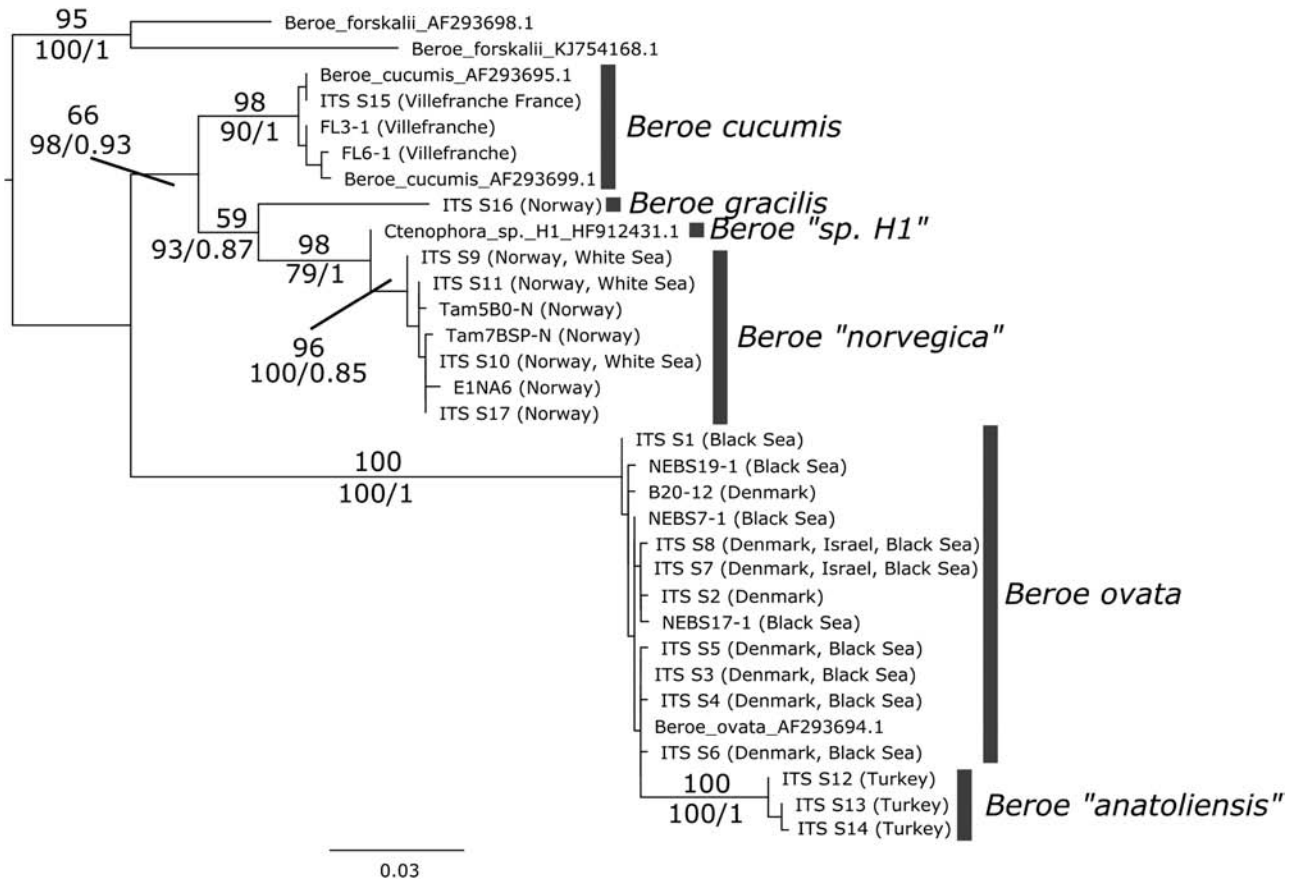

Figure 2. Internal Transcribed Spacer (ITS) maximum-likelihood tree. Sequences from GenBank include accession numbers. Sequences from this study include sampling locations. Individuals with identical sequences are represented by their sequence ID (see Supplementary Table 2 for details). Maximum-likelihood bootstrap values >50% for major clades are indicated above the lines. Neighbor-joining bootstrap values >50% and Bayesian posterior probabilities >0.50 are indicated below the line, separated by a /. Scale bar is measured in substitutions per site.

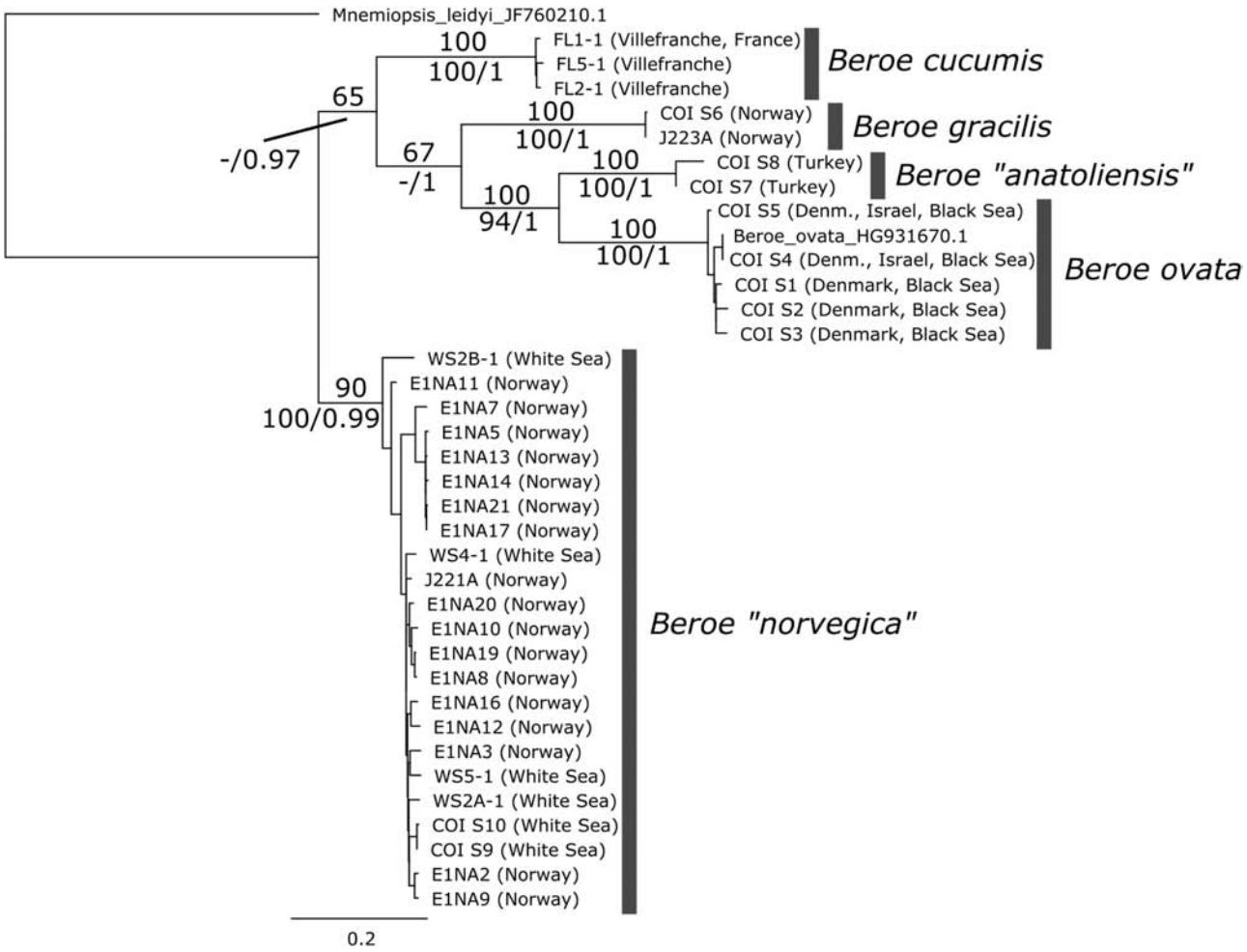

Figure 3. Cytochrome oxidase I (COI) maximum-likelihood tree. Sequences from GenBank include accession numbers. Sequences from this study include sampling locations. Individuals with identical sequences are represented by their sequence ID (see Supplementary Table 2 for details). Maximum-likelihood bootstrap values >50% for major clades are indicated above the lines. Neighbor-joining bootstrap values >50% and Bayesian posterior probabilities >0.50 are indicated below the line, separated by a /. Scale bar is measured in substitutions per site.

Table 2). Sequences are available in GenBank with Accession numbers MH217588-MH217677 (COI) and MH220085-220191 (ITS).

We identified a total of 5 well-supported clades in our COI (Figure 3) and ITS (Figure 2) trees, based on a bootstrap support cut-off of >70%. These clades most likely correspond with 5 species based on average differences between sequences and similarity to published *Beroe* ITS and COI sequences, as well as on comparisons with the average within-species sequence similarity observed between *M. leidyi* sequences (ITS mean: 99.6 ± 0.2 (standard deviation [SD]); COI mean: 99.2 ± 0.8). Our ITS and COI phylogenetic trees were concordant, geographically consistent, and largely consistent with morphological identifications. Results were very similar whether analyses were performed with all individuals separate or grouped by identical sequence. For clarity of presentation, we will only discuss results with identical sequences grouped.

Both trees include a well-supported clade (90%/0.99 and 100%/1.0 ML bootstrap support/Bayesian posterior probability for the ITS and COI trees, respectively) that includes published *B. ovata* sequences as well as all of our samples collected from the Baltic Sea (Denmark), the Levantine Sea (Israel), and the Black Sea. Within the ITS tree, sequences in this clade had an average of 99.1 ± 0.4 (SD)% sequence identity, whereas the COI tree sequences had an average of 97.3 ± 0.8 (SD)% sequence identity. The broad geographic distribution of sequences within this clade are consistent with the known introduced status of *B. ovata* in Europe. COI sequences within the *B. ovata* clade shared an average of 83.8 ± 0.5 (SD)% sequence identity with sequences in the nearest-neighbor (*B. "anatoliensis"*) clade, whereas, for ITS, sequence identity averaged 95.4 ± 0.5 (SD)%, although here *B. "anatoliensis"* formed a distinct subclade within the *B. ovata* clade.

As described above, the nearest neighbor to (COI) or within (ITS) the *B. ovata* clade is a distinct and well-supported group (100% bootstrap support/1.0 posterior probability), here preliminarily labeled as *Beroe "anatoliensis"*, consisting of samples collected from the Southern Aegean coast of Turkey. ITS sequences in this clade shared an average 99.4 ± 0.3 (SD)% sequence identity, whereas they only shared 95.4% average sequence identity with sequences in the *B. ovata* clade. The 2 sequences in the corresponding clade on the COI tree had 96.4% sequence identity to one another and 83 ± 0.5 (SD)% average sequence identity to those in the *B. ovata* clade.

The remaining sequences group into a single large clade with lower statistical support (66% bootstrap support/0.93 posterior probability) in the ITS tree, containing 3 or 4 subclades that likely correspond to 3 or 4 distinct species. Three of these species,

B. cucumis, *B. gracilis*, and a putatively undescribed *Beroe* species found in Norwegian waters and the White Sea (and labeled by us as *B. "norvegica"*), also appear as distinct clades in the COI tree. However, relationships between the clades are slightly different (i.e. uniting *B. cucumis* and *B. gracilis* with *B. ovata*/*B. "anatoliensis"* instead of *B. "norvegica"*) and basal branches have low bootstrap support in the COI tree (Figure 3).

All the samples collected from the Mediterranean (Villefranche sur Mer) formed a single well-supported clade in both ITS and COI trees (98% and 100% bootstrap support, respectively; 1.0 posterior probability). In the ITS tree, this clade is identifiable as *B. cucumis* as it includes published sequences for that species. Sequences within the *B. cucumis* clade had an average of 98.8% sequence identity for both COI (±0.1 [SD]) and ITS (±0.5 [SD]). In the ITS tree, the *B. cucumis* clade shared 88.1 ± 1.0 (SD)% sequence identity on average with its nearest-neighbor clade, containing *B. gracilis*, *Beroe* "sp. H1", and *B. "norvegica"* and 82.5 ± 1.0 (SD)% average sequence identity with the *B. ovata*/*B. "anatoliensis"* clade. In the COI tree, *B. cucumis* sequences shared between 81.1 ± 1.7 (SD)% sequence identity with sequences in the *B. gracilis*/*B. "anatoliensis"*/*B. ovata* clade and 84.9 ± 0.7 (SD)% sequence identity with sequences in the *B. "norvegica"* clade.

Three individuals collected in Norway and morphologically, identified as *B. gracilis* based on size, shape, and a lack of diverticulae, had identical ITS sequences and formed a strongly supported clade in the COI tree (100% bootstrap support/1.0 posterior probability). In the ITS tree, this sequence was part of a clade with *Beroe "sp. H1"* and *B. "norvegica"* (59% ML bootstrap support, 0.85 posterior probability), with the *B. cucumis* clade as the nearest neighbor. The *B. gracilis* ITS sequence shared 86.6 ± 0.1 (SD)% average sequence identity with sequences in the *B. cucumis* clade and 91.6 ± 1.1 (SD)% average sequence identity with samples in the *Beroe* "sp. H1" and *B. "norvegica"* clade. Relationships between species are slightly different in the COI tree, where *B. gracilis* is part of a clade with low bootstrap support (67%) and which includes *B. "anatoliensis"* and *B. ovata*, again with *B. cucumis* as the nearest-neighbor clade. Within the COI tree, the 2 sequences in the *B. gracilis* clade had 99.8% sequence identity, shared between 80.4 ± 1.2 (SD)% sequence identity with samples in the *B. "anatoliensis"*/*B. ovata* clade, and shared 84.1 ± 0.6 (SD)% sequence identity with *B. cucumis* samples.

The remaining samples, collected in Norway and the White Sea, formed a single clade with 90%/0.99 (COI) and 98%/1.0 (ITS) ML bootstrap support/posterior probability and are labeled here as *B. "norvegica"*. This clade may also include the previously published *Beroe* "sp. H1" ITS sequence (Majaneva and Majaneva 2013), although *B. "norvegica"* samples all group together in a distinct clade. *B. "norvegica"* sequences within the clade had an average of 99.7 ± 0.2 (SD)% sequence identity and shared 98.5 ± 0.3 (SD)% average sequence identity with *Beroe "sp. H1"*. *B. "norvegica"*/*"sp. H1"* ITS sequences shared an average of 91.6 ± 1.1 (SD)% sequence identity with the *B. gracilis* sequence. Within the COI tree, sequences in the *B. "norvegica"* clade shared an average of 95.9 ± 1.6 (SD)% sequence identity with one another. The *B. "norvegica"* clade differed considerably from any of the 3 other species in the COI tree, with an average sequence similarity of 81.8 ± 2.0 (SD)% to the combined set of other species. This compares to the level of difference seen when comparing *B. "norvegica"* and *M. leidyi* (GenBank Accession No. JF760210.1), where the average sequence similarity is 78.7 ± 0.6 (SD)%.

The 2 *B. forskalii* sequences used as outgroups in the ITS analysis only shared 84.6% sequence identity. As these samples were collected from France (Genbank Accession No. KJ754168.1; Simion et al. 2015) and California (AF293698.1; Podar et al. 2001), it is likely that these 2 sequences represent another case of cryptic or misidentified species.

Morphological Analysis

All *Beroe* have 8 meridional and 2 paragastral canals, which arise from the funnel and extend down the middle of the broad sides of the ctenophore. Most of them are pink in color, especially along the meridional canals and comb-rows, except for *B. gracilis*. Most *B. gracilis* are milky coloured, although some adult individuals may be slightly pink. The 8 meridional canals may have side branches, or diverticulae, extending outwards into the plane of the body surface, and some species have fusions, or anastomoses, of these diverticulae (Table 1).

Specimens from the Black Sea and Levantine Sea (Israel coastal waters) were identified as *B. ovata* alive (Black Sea) or from pictures (Israel). We did not observe alive or photographed specimens from the Southern Aegean Sea (Gokova Bay, Turkey). Specimens from France were identified as *B.cucumis* sensu Mayer from pictures. Specimens from the White Sea were identified as *B.cucumis* from pictures. About half of the specimens from Norway were initially identified as *B. ovata*, with the rest identified as *B. cucumis* and 3 morphologically identified as *B. gracilis* based on body size, shape, and a lack of diverticulae (see also Molecular Analysis, above).

Most samples from the region around Norway, Svalbard, and the White Sea formed a distinct clade, which seemed to be an undescribed species (initially described incorrectly as *B. cucumis*) and which we have provisionally named *B. "norvegica"*. Morphological analysis of some of these Norwegian specimens prior to preservation suggests that *B. "norvegica"* has an oval body shape near the aboral end and rectangular or slightly oval at the oral end. *B. "norvegica"* is much less flattened in the paragastral plane than *B. ovata*. The 8 meridional canals lay under 8 rows of ciliary combs, which extend about 3-quarters of the distance from the apical sense organ towards the mouth. Meridional canals have numerous diverticulae, which may branch out in adult ctenophores (Figure 4a), but do not anastomose in most specimens, and do not connect with paragastral canals (Figure 4a). Two oval polar plates surround the sense organ at the aboral pole and are fringed with a row of short, branched papillae. *Beroe* collected from Norway varied somewhat in morphology. Some specimens seemed to have a few anastomosing diverticulae between 2 meridional canals (but not with paragastral canals) and the shape of the diverticulae also differed between specimens. In young specimens, they were short and smooth (Figure 4a); in adults, they were long and highly branched (thorny in appearance; Figure 4b). General morphological features of studied representatives of *Beroe* are given in Table 1.

Discussion

Our ITS and COI barcoding approach revealed 5 genetic lineages of *Beroe spp.* in the seas of Europe. The lineages were geographically coherent, statistically well-supported, and consistent between genetic markers, supporting the conclusion that these results are robust. Levels of genetic differentiation between clades (p-distances ranging from approximately 10–20%, depending on comparison and marker) are broadly comparable to those of Alamaru et al. (2017), where comparisons were performed between different species in the family Coeloplanidae and where K2P distances between species clades averaged 10%. Of the lineages identified, three could

Table 1. Morphological features of studied *Beroe* (from Mayer 1912; Greve et al. 1976, Mianzan 1999; Shiganova and Malej 2009; Shiganova et al. 2007)

Morphological features	*B. cucumis sensu Mayer*	*B. gracilis*	*B. "norvegica"*	*B. ovata sensu Mayer*
Adult length (mm)	80–150	<30	>50	50–160
Ratio of length to width (l/w)	2.12–2.5	1.9–2.3	1.18–1.68	1.27–1.33
Body shape	Oval from oral end, narrowing to rounded aboral end	Slender, cylindrical body, oval at oral end, wider in oral end in adults	Oval in aboral end, only slightly oval or nearly straight in oral end	Wide miter-shaped body, aboral end is rounded, oral end nearly flat and can be wider than the body width in adults
Flattening in the paragastral plane	Not flattened	Moderately flattened	Slightly flattened	Very flattened
Color	Pink	Milky or slightly pink	Pink	Pink to brownish in large adults
Diverticulae in Meridional Canals	Numerous diverticulae with side branches in adults, but diverticulae do not anastomose with each other and do not connect with paragastral canals	Few or absent	Numerous diverticulae with side branches in adults, but diverticulae do not anastomose in most specimens and do not connect with paragastral canals	Meridional canals have a network of anastomosing diverticulae which connect with paragastral canals
Structure of Aboral Pole	Polar plate surrounds the sense organ at the aboral pole and is fringed with a row of long, branched papillae	Aboral pole is fringed with a row of papillae	Two oval polar plates surround the sense organ at the aboral pole, which are fringed with a row of short, branched papillae	The polar plate surrounding the sense organ at the aboral pole is not fringed with a row of branched papillae

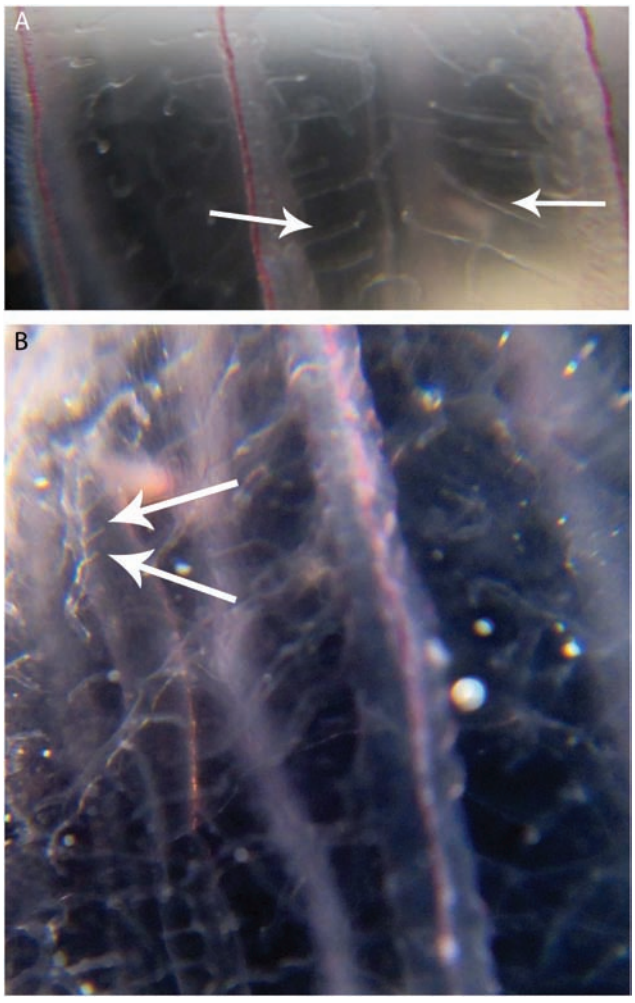

Figure 4. (a) *Beroe "norvegica"* morphotype 1, with short, smooth diverticulae (indicated by arrows). (Photo by H. Ringvold). (b) *Beroe "norvegica"* morphotype 2, with long, thorny diverticulae (indicated by arrows). (Photo by H. Ringvold).

be assigned to the known species *B. cucumis* and *B. ovata* based on similarity to previously published sequences from GenBank, and one most likely represents *B. gracilis* based on DNA sequence distinctiveness and morphological identification, specifically their small size and lack of diverticulae. The 2 other lineages might reflect new species *Beroe "norvegica"* and *B. "anatoliensis"*, but confirmation requires further study.

As a group, Ctenophora seem to be particularly difficult to identify morphologically, leading to a high degree of uncertainty about the true number of ctenophore species (Mills 2018). Mills (2018) has argued that many species names are likely to be synonymized with further study, and we believe that there are likely to be some widespread species (such as *B forskalii*, described above) that consist of 2 (or more) cryptic species. As previously mentioned, this likely results from a lack of distinguishing morphological characters, limited knowledge of their taxonomy, and difficulty in sampling and preserving specimens (Purcell 2009; Licandro et al. 2015). Given the challenges of identifying ctenophores morphologically, molecular tools using ITS and COI sequences represent a powerful alternative method for identifying ctenophores to species. However, identifications are currently limited by the marked lack of ITS and COI sequences in publicly-available databases. Illustrating this problem, a search of the NCBI GenBank "Nucleotide" database on 8 June 2017 using keywords "*Beroe*" and "internal transcribed" and filtering results for animals returned sequences for 4 known species (of a putative 25 *Beroe* species worldwide): *B. cucumis* sensu Mayer, *B. ovata* sensu Mayer, *B. forskalii*, *B. abyssicola*, and 2 undescribed species. A similar search replacing "internal transcribed" with "cytochrome oxidase" returned a single sequence for *B. ovata*. Searching for "*Beroe*" on the Barcode of Life Database (www.boldsystems.org) returned 17 specimens in 3 species (*B. abyssicola*, *B. cucumis*, *B. ovata*), none of which have associated DNA sequence data. Thus, there exists a major gap in baseline data for using DNA barcodes to identify unknown ctenophores. Almost all known species of *Beroe* remain to be barcoded, and our naming of 2 new species based on sequence distinctiveness should be considered preliminary. That said, our results do shed some light on the distributions of both native and introduced *Beroe* spp. in European seas.

Black and Mediterranean Seas

Our data confirm that *B. ovata* sensu Mayer has now joined *M. leidyi* as a widespread introduced ctenophore in the seas of Europe. *B. ovata* was initially identified morphologically, and these identifications were previously supported using 18S molecular data (Konsulov and Kamburska 1998; Shiganova et al. 2001; Bayha et al. 2004). Our molecular and morphological results confirm previous identifications and demonstrate that the species is widespread in Europe, including waters off Denmark, Israel, and in the Black Sea (Shiganova et al. 2014a, b).

We collected and morphologically identified 6 *B. cucumis* samples from the Mediterranean Sea at Villefranche sur Mer, France. ITS and COI sequences for these French samples formed a single well-supported clade in both trees. Reference COI sequences for *B. cucumis* were not available. However, our ITS sequences were very similar to *B. cucumis* reference sequences from the east and west coasts of North America. This result supports morphological identifications and lends credence to the hypothesis that *B. cucumis* is broadly distributed in warm seas. *B. cucumis* has previously been observed and identified in swarms of *M. leidyi* in the northern Adriatic Sea, the Levantine Sea, and in the Mediterranean (Shiganova and Malej 2009; Galil and Gevili 2013).

Another set of specimens, collected from the Southern Aegean coast of Turkey (Gokova Bay), were previously identified morphologically as introduced *B. ovata*, which were assumed to have arrived from the Black Sea (Gulsahin and Tarkan 2013). However, our genetic analyses suggest that these specimens are actually the nearest-neighbor clade to *B. ovata* and are sufficiently different in both COI and ITS sequences that they likely represent a different species. Although we have preliminarily labeled these specimens as *B. "anatoliensis"* after the region, no reference sequence data exists for *B. mitrata* (previously recorded in the Mediterranean), so whether these samples represent a described species, an undescribed native species, or an introduced species is unclear.

North Sea/Arctic Ocean

Two species, *B. gracilis* Künne, 1939 and *B. cucumis* Fabricius, 1780, were previously thought to occur as natives in the North Sea (Greve et al. 1976). The former species is known from the North Sea and Skagerrak (Greve et al. 1976; Hansson 2006; Granhag et al. 2012). The latter species was first described from Greenland by Fabricius (1780), was subsequently reported as a cosmopolitan species (Moser 1909), and is the only *Beroe* previously known to occur in the Baltic Sea. *B. cucumis* has been reported in the Danish marine fauna for more than 100 years, commonly occurring throughout the area (Mortensen 1912; Kramp 1915; Shiganova et al. 2014b). Previous work on samples from Danish waters based on morphology and 18S sequences (Shiganova et al. 2014b), identified *B. ovata* (sensu Mayer) and *B. cucumis*, both with close sequence matches to specimens collected from North America: *B. ovata* (JN653095, from Tampa Bay, FL, Daniels and Breitbart 2012; AF293694, from Woods Hole, MA, Podar et al. 2001), and *B. cucumis* (AF293695, from Gulf Stream, FL, and AF293699, from Santa Barbara, CA, Podar et al. 2001). Given the limited phylogenetic signal in 18S sequences in ctenophores, these identifications should be considered uncertain. However, we identified the same *B. ovata* in our samples, supporting the previous identifications of that species and confirming its spread north to Denmark. We failed to identify any *B. cucumis* in our Danish samples, probably because the species was not sampled, although the possibility of a previous misidentification cannot be ruled out.

We re-analyzed 5 samples previously described in Ringvold et al. (2015). Three of these samples were morphologically identified in that study as *B. gracilis* mainly based on color, size, and absence of diverticulae of meridional canals, supported by the 99.8% similarity of their 18S sequences to previously published *B. gracilis* sequences from Podar et al. (2001). This represented a first identification of this species from Norwegian waters. The identification of these samples as *B. gracilis* is consistent with our ITS and COI analyses. The 3 samples had identical ITS sequences and formed strongly supported and distinct clades in both ITS and COI trees. Based on previous morphological identifications, we suggest that these individuals were likely *B. gracilis*, but a lack of reference ITS and COI sequences for that species hinders definitive molecular identification. The remaining 2 samples from Ringvold et al. (2015), which were previously identified morphologically and based on 18S sequences as *B. cucumis*, formed a well-supported clade in both neighbor-joining trees with 26 new samples collected from Norway and the White Sea. Sequences for both ITS and COI from these samples, most of which were initially identified morphologically as *B. cucumis* and *B. ovata* based on meridional canals with and without anastomosing side branches, differed markedly from known sequences from those 2 species. We have tentatively named this clade *B. "norvegica"* in recognition of the high level of sequence distinctiveness and described the morphology of the samples collected, but confirmation of the existence of this new species will require additional sampling and analysis. As *B. cucumis* has previously been described from the North Sea, our failure to find any examples of that species in Norway is noteworthy, given our relatively large number of samples. This raises the possibility that previous descriptions of *B. cucumis* from the North Sea and Arctic might represent examples of *B. "norvegica"*, although this will require additional confirmation.

Conclusions

Barcoding revealed 5 genetic lineages of *Beroe* in the seas of Northern and Southern Europe, 3 of which could be assigned to the known species *B. cucumis, B. gracilis,* and *B. ovata*. The other 2 lineages might reflect new species, but confirmation of this requires further study. We have preliminarily named them *B. "norvegica"* and *B. "anatoliensis"* based on the regions where the collections were made. Our integrative approach combining DNA barcoding with morphological study facilitated easy species identification and reduced taxonomic confusion in *Beroe*. Given the apparent lack of fine-scale phylogenetic signal in 18S sequences, we would recommend COI and ITS as appropriate barcoding regions for *Beroe* species. Given that ctenophores are transported globally and some are highly invasive, the presence of cryptic or undescribed species poses a real problem. The arrival of *B. ovata* in Europe and the positive impact of its control of the aggressive invader *M. leidyi* represents something of a lucky break. As this kind of good fortune may not accompany additional ctenophore introductions, it is critical to understand the worldwide diversity, distributions, trophic interactions, and potential transport of these ecologically important but understudied organisms.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

Funding

Natural Sciences and Engineering Research Council of Canada Discovery Grant and Canada Research Chair to H.J.M.; FASO Russia (theme No. 0149-2018-0009) to T.A.S.

Acknowledgments

We are extremely grateful to K. Stefanova, N. Gulsahin, D. Angel, B. Galil, F. Lombard, and G. Abysova for collecting specimens from different regions for this project. We also thank S. Ghabooli, S. Nolan, and K. Quenneville for assistance with lab work. The manuscript was significantly improved by comments from M. Donovan, 2 anonymous reviewers, and the editor.

Data Availability

All COI and ITS sequences have been deposited in GenBank (Accession nos. MH217588-MH217677 [COI] and MH220085-220191 [ITS]).

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